

## Live Cell Identification of Redox Regulated Dithiol Sensors by Chemical Probes

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**Project Goals: The long-term goal of the PNNL BSFA is to develop a predictive understanding of pathways and regulatory schemes involved in solar energy conversion to biofuel precursors or products. To date, knowledge gaps are not limited to biochemical pathways of energy and carbon flux but to an even larger degree include regulatory events within and between metabolic subsystems that include intracellular signals to which transcriptional regulation is responding. We suggest that “redox sensing,” as a means to maintain redox homeostasis in photosynthesizing cells, is likely an equally important mechanism. In this project, we are investigating the central hypothesis that these sensors regulate electron flux and carbon partitioning in cyanobacteria. Specifically, we have developed and applied probe approaches for live cell capture, characterization, and imaging of redox sensors of environmental change using advanced separations, mass spectrometry and traditional biochemical techniques. It is anticipated that the identification of redox-sensitive dithiol linkages and their modulation by thioredoxin (Trx), peroxiredoxin, and other regulators will provide key inputs for understanding the control points of flux distribution.**

The primary challenge in identifying redox-regulated dithiol sensors *in vivo* is that cysteine residues are highly reactive and easily oxidized to dithiol linkages following cellular lysis. This eliminates the ability to experimentally measure redox regulation within native physiological settings, and makes requisite the exogenous addition of chemical or biological reductants. Therefore, we developed two different but complementary approaches for synthesizing and employing cell permeable probe reagents that react in live cells (*in situ*) with thiols and permit real-time imaging and mass spectrometric characterization of probe targets. In the first approach we have synthesized click-chemistry enabled chemical probes for fluorescent and mass spectrometric identification of redox regulated dithiol sensors, and in the second approach we used a cell-permeable arsenic probe (TRAP-Cy) that selectively binds to reduced disulfides in close proximity to trap available dithiols in living cells prior to cell lysis. We have used our probes *in situ* to identify proteins in *Synechococcus* sp. PCC 7002 that under-go disulfide exchange in response to changes in cellular conditions.

**Click-Chemistry Enabled Probes.** Probes were synthesized with three chemical elements: a moiety to impart cell permeability, an iodoacetamide or maleimide group for irreversibly labeling cysteines, and a reporter tag for detection and isolation of probe-labeled proteins. We exploited the multimodal bio-compatible click chemistry (CC) reaction to create “tag-free” probes for profiling proteins in living systems. Probe-labeled proteins were visualized by addition of a complementary azido-tetramethylrhodamine (fluorescence) or an azido-biotin tag for enrichment and mass spectrometric analysis (LC-MS).

The maleimido and iodoacetamide probes were added simultaneously *in vitro* or *in situ* to *Synechococcus* sp. PCC 7002 grown in a turbidostat under maximal growth rate conditions. Cells were also removed and placed in the dark for two hours and then labeled *in vitro* or *in situ*. Following probe-labeling cells were lysed and probe-labeled proteins attached to biotin via CC. The probe-labeled proteins were then enriched on streptavidin, digested with trypsin, and the peptides analyzed by LC-MS. Critically, in the cells that were labeled post-lysis (*in vitro*) we found no changes in redox regulation of dithiol sensors, demonstrating that lysis rapidly oxidizes biological samples. However, when we analyzed the *in situ* labeled cells we identified 153 redox-regulated proteins that were statistically different between the light and dark conditions. In a follow-up study using a carbon-limited turbidostat we were able to identify redox changes *in situ* within 30 seconds following the addition of CO<sub>2</sub>. A time-course study revealed remarkable changes from 30 seconds to 60 minutes post CO<sub>2</sub> addition. Importantly, these changes cannot be measured by traditional transcriptomic or proteomic measurements because these redox events occur within a time-frame that protein and mRNA content has not changed.

Arsenic Probes. TRAP-Cy contains a cyanine dye to facilitate high-throughput gel-based screening prior to affinity purification and MS based identification of reactive dithiols. Using this trapping reagent, we have explored redox-active dithiol reactivity and their relationship to photosynthetic metabolism in *Synechococcus* sp. PCC 7002 under varied environmental conditions. Upon cell lysis, covalently bound cysteines in association with TRAP-Cy are stable, permitting reduction of all remaining thiols and their alkylation. After treatment with excess ethane dithiol, the TRAP-Cy probe is released, permitting enrichment of these cysteines using a thiol-capture affinity resin. In the vast majority of cases homology models of the structures demonstrate that identified thiols are in close proximity in the tertiary structure. Consistent with our hypothesis that dithiol “switches” (e.g., Cys-Xxx<sub>n</sub>-Cys) activated by thioredoxin-dependent pathways regulate electron flux and carbon sequestration to modulate energy partitioning.

Probe-identified proteins from both probe types map well onto multi-subunit supramolecular complexes involving photosynthetic pathways associated with efficient collection of excitation energy (light harvesting), electron transfer reactions linked to formation of electrochemical gradients, carbon dioxide sequestration (dark reactions), and ATP synthesis. Additional redox-dependent pathways include those involving chaperone activity, transcriptional regulation, and antioxidant proteins linked to protein repair. Together, these results provide quantitative information regarding redox-dependent switches associated with photosynthetic regulation, and provide a systems biology tool capable to providing high-throughput information necessary for predictive metabolic modeling. Finally, both cell permeable probe approaches represent the only existing methods for identifying and imaging live cell redox regulation, and they will be critical to informing the predictive models of metabolism needed for bioenergy applications.

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